Psoriasis Is Characterized by Accumulation of Immunostimulatory and Th1/Th17 Cell-Polarizing Myeloid Dendritic Cells

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Myeloid dermal dendritic cells (DCs) accumulate in chronically inflamed tissues such as psoriasis. The importance of these cells for psoriasis pathogenesis is suggested by comparative T-cell and DC-cell counts, where DCs outnumber T cells. We have previously identified CD11c$^+$-blood dendritic cell antigen (BDCA)-1$^+$ cells as the main resident dermal DC population found in normal skin. We now show that psoriatic lesional skin has two populations of dermal DCs: (1) CD11c$^+$BDCA-1$^+$ cells, which are phenotypically similar to those contained in normal skin and (2) CD11c$^+$BDCA-1$^-$ cells, which are phenotypically immature and produce inflammatory cytokines. Although BDCA-1$^+$ DCs are not increased in number in psoriatic lesional skin compared with normal skin, BDCA-1$^-$ DCs are increased 30-fold. For functional studies, we FACS-sorted psoriatic dermal single-cell suspensions to isolate these two cutaneous DC populations, and cultured them as stimulators in an allogeneic mixed leukocyte reaction. Both BDCA-1$^+$ and BDCA-1$^-$ myeloid dermal DC populations induced T-cell proliferation, and polarized T cells to become T helper 1 (Th1) and T helper 17 (Th17) cells. In addition, psoriatic dermal DCs induced a population of activated T cells that simultaneously produced IL-17 and IFN-γ, which was not induced by normal skin dermal DCs. As psoriasis is believed to be a mixed Th17/Th1 disease, it is possible that induction of these IL-17$^+$IFN-γ$^+$ cells is pathogenic. These cytokines, the T cells that produce them, and the inducing inflammatory DCs may all be important new therapeutic targets in psoriasis.

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INTRODUCTION

Psoriasis is a common chronic inflammatory skin disease, which results in great morbidity for severely affected patients. In recent years, much progress has been made in understanding the pathogenesis and treatment of this disease. We now appreciate that psoriasis results from complex interactions between T cells, dendritic cells (DCs), and keratinocytes (Lowes *et al.*, 2007). Until recently, psoriasis has been considered a classical type 1 autoimmune disease, with a strong IFN-γ T helper 1 (Th1) signal. However, a new subset of T cells, T helper 17 (Th17) cells, have now been described in murine models of autoimmune inflammation (Weaver *et al.*, 2007), and we have reported the presence of these cells in psoriasis (Lowes *et al.*, 2008). Th17 cells produce IL-17 and IL-22, and have other important downstream pro-inflammatory effects in skin (Liang *et al.*, 2006; Zheng *et al.*, 2007). DCs may be very central pathogenic players in psoriasis, both by activating T cells and by producing amplifying cytokines and chemokines during inflammation. In the skin, the main DC populations include epidermal DCs (Langerhans cells) and dermal DCs (myeloid DCs and plasmacytoid DCs). We were interested in further characterizing dermal myeloid DCs in psoriasis, as these cells may be an important therapeutic target.

Recently, we described that the best marker for identifying dermal myeloid DCs in normal skin is CD11c (Zaba *et al.*, 2007b). Also, we have previously reported that there is a large increase in CD11c$^+$ cells in psoriasis (Abrams *et al.*, 1999; Gottlieb *et al.*, 2005; Lowes *et al.*, 2005). These CD11c$^+$ cells include a subset of inflammatory DCs called tumor necrosis factor (TNF)- and inducible nitric oxide synthase (iNOS)-producing DCs (Tip-DCs) (Lowes *et al.*, 2005). Tip-DCs were first described in the spleen during a murine model of *Listeria monocytogenes* infection (Serbina *et al.*, 2003; Tam and Wick,

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Abbreviations: allo-MLR, allogeneic mixed leukocyte reaction; BDCA, blood dendritic cell antigen; CFSE, carboxy fluoroscein succinimidyl ester; DC, dendritic cell; DC-LAMP, dendritic cell–lysosomal-associated-membrane protein; DC-SIGN, dendritic cell-specific ICAM-3-grabbing nonintegrin; iNOS, inducible nitric oxide synthase; Th1, T helper 1; Th17, T helper 17; Tip-DC, tumor necrosis factor- and iNOS-producing dendritic cell

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2004). Pathogenicity of these Tip-DCs in psoriasis is suggested by the rapid downmodulation of Tip-DC products TNF, iNOS, IL-20, and IL-23 during effective treatment with TNF-blocking drugs (Zaba *et al.*, 2007a).

In normal skin, CD11c$^+$ cells are nearly all blood dendritic cell antigen (BDCA)-1$^+$ (Zaba *et al.*, 2007b). BDCA-1 is also known as CD1c, and this molecule is part of the CD1 family of invariant major histocompatibility complex molecules that are important in presentation of lipid antigens to T cells (Barral and Brenner, 2007). In this article, we show that unlike normal skin, most of these CD11c$^+$ cells in psoriatic plaques are actually BDCA-1$^-$. They are relatively immature DCs, with little dendritic cell–lysosomal-membrane-associated protein (DC-LAMP) and DEC-205/CD205 coexpression in psoriatic lesions. Hence, *in situ* there were two main types of dermal DCs in psoriasis lesions: CD11c$^+$BDCA-1$^+$-resident DCs and CD11c$^+$BDCA-1$^-$ inflammatory DCs. Dermal single-cell suspensions for phenotype analysis and functional studies showed that both populations were allo-stimulatory and were able to polarize allogeneic T cells into IL-17-producing Th17 cells.

RESULTS

Psoriatic myeloid dermal DCs are CD11c$^+$BDCA-1$^-$BDCA-3$^-$

To quantify cells in each dermal DC compartment, we performed immunohistochemistry on normal skin and psoriasis paired lesional/nonlesional samples ($n=20$) (Figure 1). Both nonlesional and lesional psoriasis samples had fivefold fewer BDCA-1$^+$ DCs ($P<0.001$) (Figure 1a and b). However, BDCA-1 cell counts did not change significantly in a group of psoriatic patients treated with etanercept (Figure 1a) (Zaba *et al.*, 2007a). There were twofold more BDCA-3$^+$ DCs compared with normal skin ($P<0.001$ and $P<0.05$, respectively) (Figure 1a and b). The BDCA-3 + antibody gave some nonspecific keratinocyte staining, as seen by others (Narbutt *et al.*, 2006), but it is currently the only available BDCA-3 antibody. In the dermis, there was a leukocyte pattern of distribution and a DC morphology with this antibody, and only dermal cells were counted. CD11c$^+$BDCA-1$^-$BDCA-3$^-$ cell numbers were calculated by subtracting BDCA-1 and BDCA-3 cell counts from CD11c cell counts. Although lesional and nonlesional psoriasis sections contained similar numbers of BDCA-1$^+$ and BDCA-3$^+$ cells, CD11c$^+$BDCA-1$^-$BDCA-3$^-$ cells were increased 10-fold in psoriasis plaques compared with nonlesional skin ($P<0.001$), and 30-fold compared with normal skin (Figure 1b) ($P<0.001$). In addition, we performed FACS on whole blood from normal ($n=6$) and psoriasis ($n=6$) subjects and found that BDCA-1$^+$ and BDCA-3$^+$ myeloid DC subsets (MacDonald *et al.*, 2002) were decreased in peripheral blood of psoriasis patients compared with normal volunteers (Figure 1c and d). Negative control staining (without primary antibody) is shown in Supplementary Figure 1e.

We next evaluated these populations *in situ* by two-color immunofluorescence. In previous studies on normal human skin, we have characterized two populations of myeloid

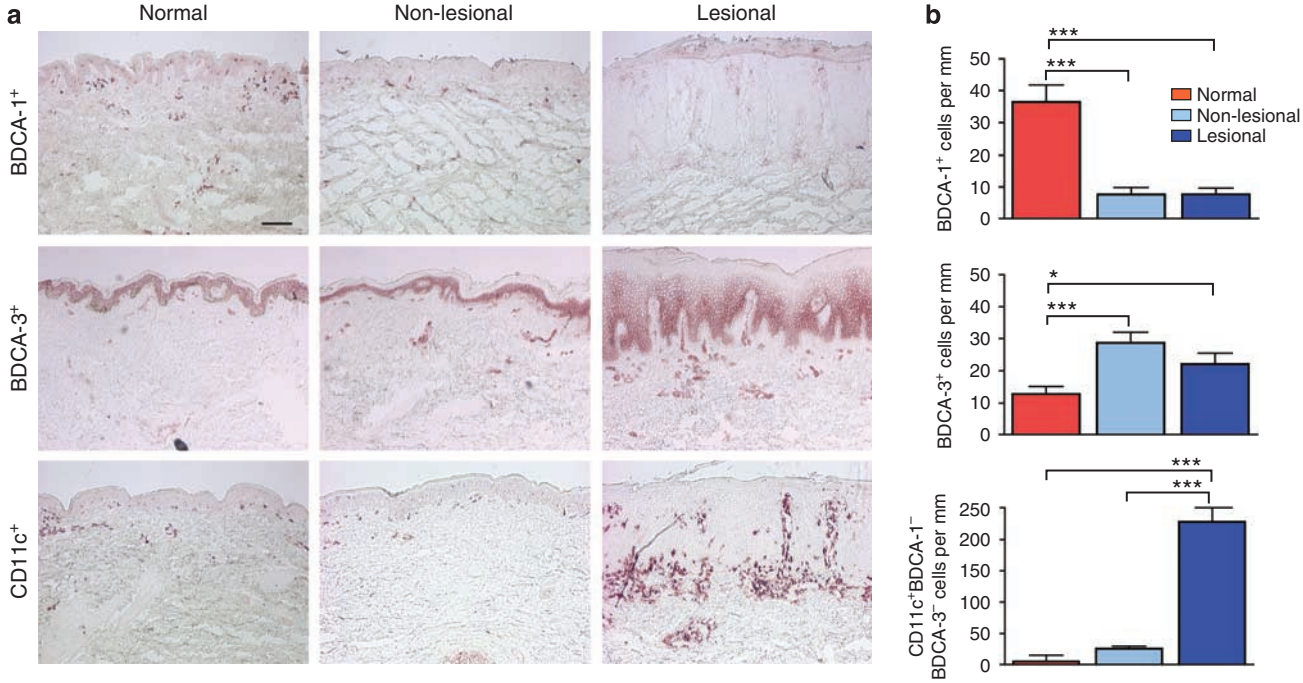

Figure 1. CD11c+ dermal DCs are the major DC population accumulating in psoriasis lesional skin. (**a**) Representative immunohistochemistry of BDCA-1$^+$ cells, BDCA-3$^+$ cells, and CD11c$^+$ cells in normal, nonlesional and lesional psoriatic skin. (**b**) Quantification of myeloid DCs per mm skin stained by immunohistochemistry of normal skin (red boxes; $n=20$), nonlesional skin (light blue boxes; $n=20$), and matched psoriatic lesional skin (dark blue boxes; $n=20$). CD11c$^+$BDCA-1$^-$BDCA-3$^-$ cell numbers were calculated by subtracting BDCA-1 and BDCA-3 cell counts from CD11c cell counts. Error bars indicate SEM. (*) $P<0.05$, (***) $P<0.001$. Bar = 100 μm.

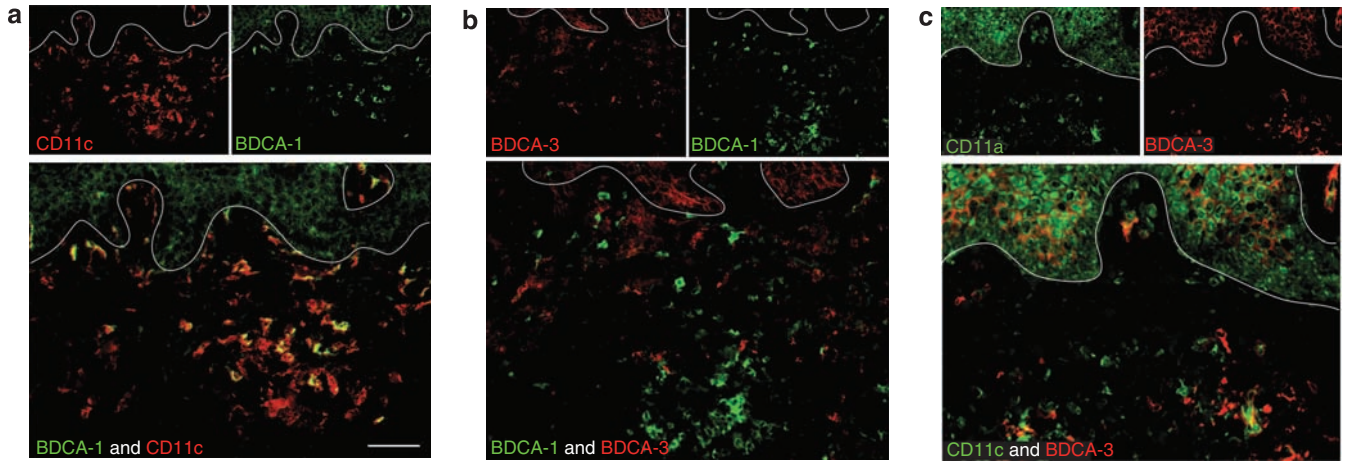

Figure 2. Most CD11c$^+$ myeloid DCs are BDCA-1$^-$ in psoriasis lesional skin. (**a**) The majority of CD11c$^+$ cells in psoriatic dermis were BDCA-1$^-$, whereas a small subset of CD11c$^+$ cells coexpressed BDCA-1$^+$. (**b**) BDCA-1 and BDCA-3 identified separate myeloid DC populations in the dermis. (**c**) Most BDCA-3$^+$ cells coexpressed CD11c, and some BDCA-3 staining was observed on blood vessels. In all immunofluorescence figures, single-stained controls are above the merged image, white line denotes dermoepidermal junction, dermal collagen fibers gave green autofluorescence, and antibodies conjugated with a fluorochrome often gave background epidermal fluorescence. Bar = 100 μm.

CD11c$^+$ dermal DCs: BDCA-1$^+$ dermal DCs comprise approximately 90% of all CD11c$^+$ dermal cells, and the remaining 10% of CD11c$^+$ cells are BDCA-1$^-$ (Zaba *et al.*, 2007b). We found that in psoriasis, there was a reversal of this ratio of BDCA-1$^+$ cells, as the minority of the CD11c$^+$ cells coexpressed BDCA-1 (Figure 2a). BDCA-1$^+$ cells aggregated together in dermal clumps (Figure 2a and b), compared with CD11c$^+$ cells, which were located mostly in the upper reticular dermis and dermal papillae. BDCA-3 identifies an additional population of myeloid DCs in the circulation (MacDonald *et al.*, 2002) and in psoriatic dermis (Figure 2b). This marker was expressed on CD11c$^+$ cells scattered throughout the dermis and also on blood vessels (Figure 2c). Similarly, in normal skin dermis, the few BDCA-3$^+$ cells that were present were CD11c$^+$, and the BDCA-1 and BDCA-3 identified discrete populations ($n = 4$) (Figure 1b). As CD11c$^+$BDCA-1$^+$ cells are the major resident dermal DC population in normal skin, the remainder of our study compares resident CD11c$^+$BDCA-1$^+$ DCs in normal skin with CD11c$^+$BDCA-1$^+$ and CD11c$^+$BDCA-1$^-$ DCs in the psoriatic inflammatory infiltrate.

CD11c$^+$ BDCA-1$^-$ myeloid dermal DCs include Tip-DC population

In psoriasis lesional tissue, the majority of CD11c$^+$ cells were iNOS producing (Figure 3a), and the cellular iNOS expression appeared punctate and intracytoplasmic. Resident CD11c$^+$ BDCA-1$^+$ dermal DCs had <10% iNOS coexpression (Figure 3b). Similarly, TNF was expressed by >90% of CD11c$^+$ cells (Figure 3c) and <20% of CD11c$^+$ BDCA-1$^+$ dermal DCs (Figure 3d). In addition, iNOS and TNF identified occasional nonlesional Tip-DCs (Figure 3e) and many lesional Tip-DCs (Figure 3f). In conclusion, Tip-DCs were identified as a subpopulation contained predominantly within the CD11c$^+$ BDCA-1$^-$ myeloid DC population.

CD11c$^+$ BDCA-1$^-$ inflammatory DCs show some expression of macrophage markers compared with BDCA-1$^+$ cells

To further characterize the CD11c$^+$ dermal DCs, we evaluated coexpression with a series of myeloid cell markers (Figures 4 and 5). Over half of CD11c$^+$ DCs expressed low-level CD14 (Figure 4a). Most CD11c$^+$ cells in the papillary dermis were CD14$^-$, whereas most in the reticular dermis were CD14^{lo}. In contrast, only occasional BDCA-1$^+$ cells expressed CD14 (Figure 4b). Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN)/CD209, a marker of both immature DCs and macrophages in normal human skin (Zaba *et al.*, 2007b), partially colocalized with CD11c (Figure 4c) but did not colocalize with BDCA-1 (Figure 4d). Similarly, CD163, a marker of normal dermal macrophages (Zaba *et al.*, 2007b), blood monocytes, and blood DCs (Maniecki *et al.*, 2006), was expressed on some CD11c$^+$ cells (Figure 4e), but not on CD11c$^+$BDCA-1$^+$ DCs (Figure 4f). In normal skin, CD11c is not expressed on CD163$^+$ macrophages (Zaba *et al.*, 2007b), despite the myeloid origin of macrophages.

Single antigens specific for mature DCs include DC-LAMP/CD208 and endocytic receptor DEC-205/CD205 (Figure 5). Nearly all DC-LAMP$^+$ and DEC-205$^+$ cells were in dermal aggregates, and coexpressed CD11c and BDCA-1. Although most BDCA-1$^+$ cells coexpressed these two mature DC markers, there were many CD11c$^+$ cells that did not, identifying BDCA-1$^-$ myeloid dermal DCs as less mature than BDCA-1$^+$ cells.

Myeloid DCs obtained from psoriatic dermis are immunostimulatory and activate Th17 and Th1 cells

Single cell suspensions of normal skin ($n = 3$) and psoriasis lesions ($n = 3$) were obtained by enzymatic splitting of the epidermis, and then culturing the dermis for 2–3 days to allow the leukocytes to migrate out of the dermal scaffold. For functional experiments, we then FACS-sorted this bulk

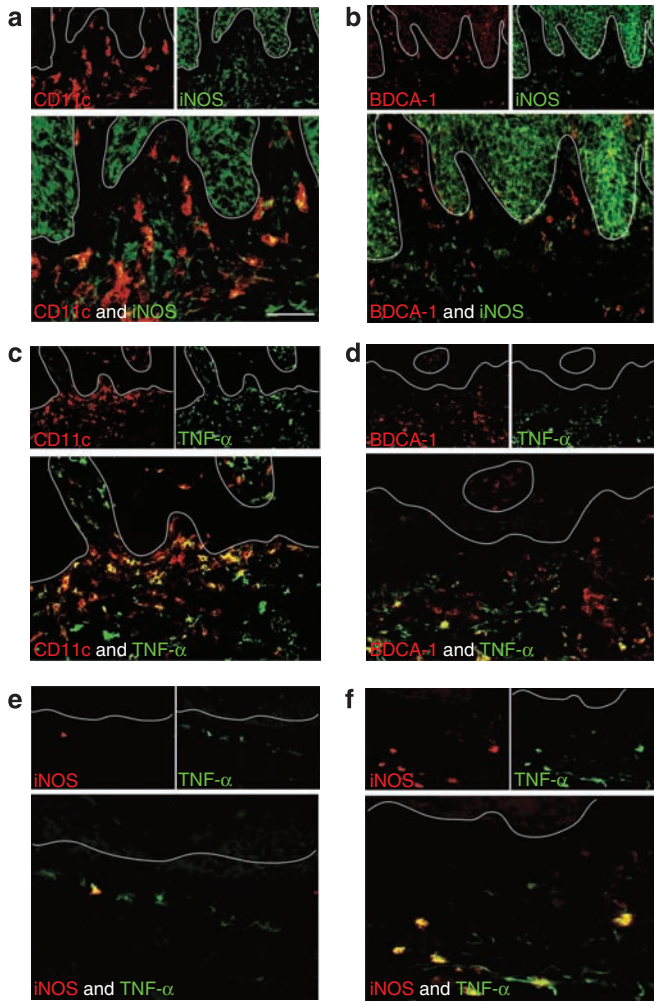

Figure 3. $CD11c^+BDCA\text{-}1^-$ DCs contain the Tip-DC population. (**a**) Most $CD11c^+$ dermal DCs in psoriatic lesional skin coexpressed iNOS compared with (**b**) BDCA-1^+ cells. (**c**) Most $CD11c^+$ dermal DCs in lesional psoriatic skin coexpressed TNF compared with (**d**) BDCA-1+ cells. Approximately, 25% of BDCA-1^+ cells coexpressed TNF. (**e**, **f**) iNOS and TNF were coexpressed in the same cell, identifying Tip-DCs *in situ*. Few Tip-DCs were observed in (**e**) psoriatic nonlesional skin compared with (**f**) lesional skin. Bar = 100 μm.

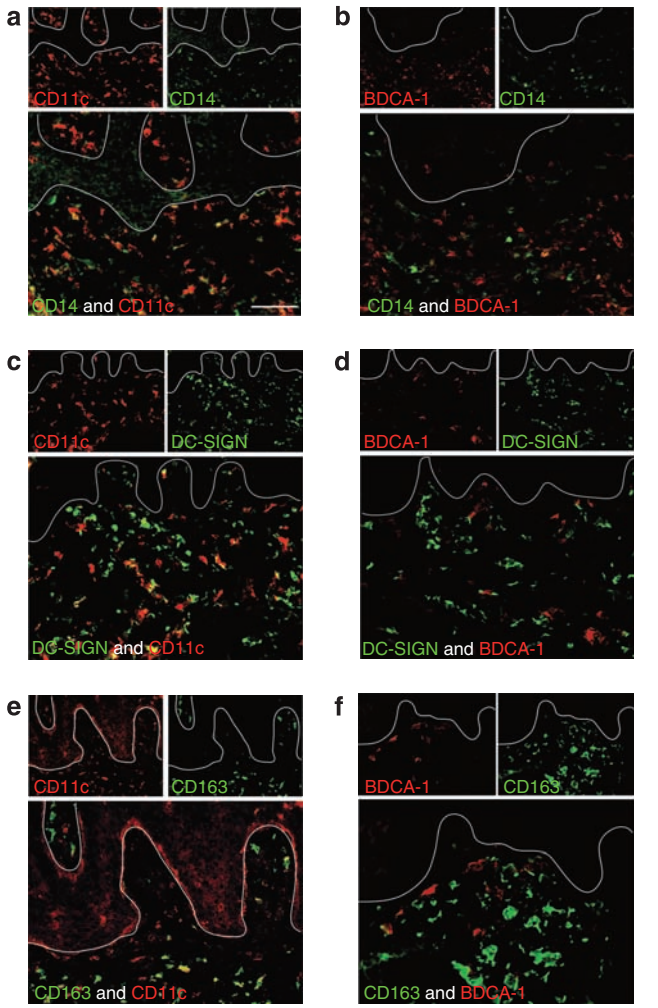

Figure 4. $CD11c^+BDCA\text{-}1^-$ inflammatory dermal DCs express CD14 and DC-SIGN. (**a**) A subset of $CD11c^+$ cells in the reticular dermis expressed low-level CD14. (**b**) Few BDCA-1^+ dermal DCs coexpressed CD14, and (**c**) 80% of $CD11c^+$ cells were DC-$SIGN^+$. This was not an exclusive myeloid DC marker, as some DC-$SIGN^+$ cells were not $CD11c^+$. (**d**) BDCA-1^+ cells did not coexpress DC-SIGN. (**e**) $CD163^+$ macrophages showed some CD11c coexpression. (**f**) BDCA-1^+ cells did not express CD163. Bar = 100 μm.

dermal single cell suspension, obtaining 10,000–50,000 cells in each population.

DC phenotype. For surface phenotyping of normal and psoriatic dermal myeloid DCs by FACS, large cells were gated on $CD11c^+HLA\text{-}DR^+$, a classic definition of a myeloid DC, and further gated according to BDCA-1 expression (Figure 6a). Phenotypic characterization of BDCA-1^+ cells from normal skin (Figure 6a, box 1) and psoriasis lesions (Figure 6a, Box 2), as well as psoriatic BDCA-1^- cells (Figure 6a, Box 3) is shown in Figure 6b and Figure 2.

$CD11c^+$BDCA-1^+-resident dermal DCs from both normal (Figure 6b, row 1) and psoriasis skin (Figure 6b, row 2) had similar levels of the lineage marker CD11c, and HLA-DR major histocompatibility complex-II protein. In contrast, $CD11c^+BDCA\text{-}1^-$ psoriatic dermal DCs (Figure 6b, row 3) had 10-fold decreased HLA-DR expression. Expression of costimulatory molecules CD83 and CD86 was highest on psoriatic $CD11c^+$BDCA-1^+ cells. Expression of the mature DC markers DEC-205/CD205 and CD208/DC-LAMP was highest on the psoriatic $CD11c^+$BDCA-1^+ cells, with a gradient of expression on psoriatic $CD11c^+BDCA\text{-}1^-$ cells, and normal skin resident $CD11c^+$BDCA-1^+ cells. CD209/DC-SIGN showed the highest expression on normal skin $CD11c^+$BDCA-1^+ cells, and was not present on BDCA-1^- psoriatic DCs (unlike *in situ*).

CD40 was low on all three populations (Figure 2). $CD11c^+BDCA\text{-}1^-$ cells from psoriatic lesions showed three- to sixfold increased CD14 LPS receptor expression and were 20% smaller than both $CD11c^+$ BDCA-1^+

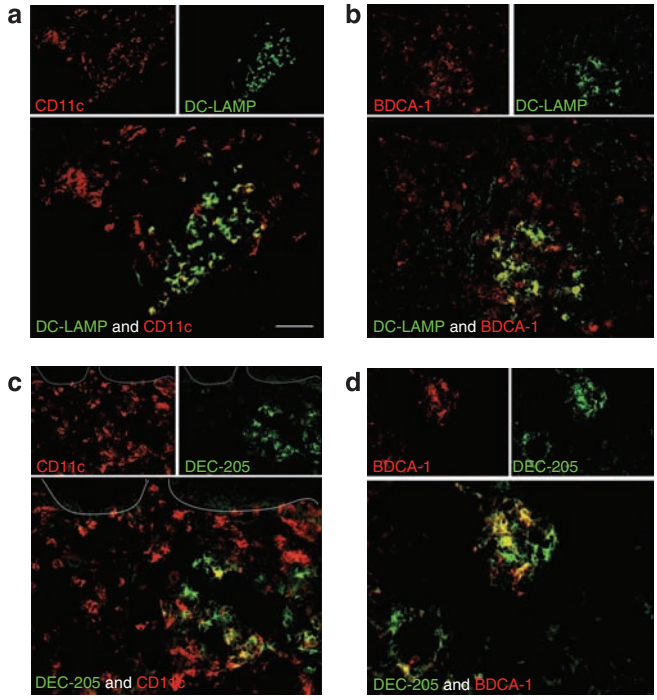

Figure 5. CD11c$^+$BDCA-1$^+$ cells are phenotypically mature dermal DCs in psoriasis. DC-LAMP and DEC-205 identified mature DCs, often located in dermal aggregates. (**a**) DC-LAMP$^+$ cells were CD11c$^+$, and (**b**) BDCA-1$^+$. (**c**) DEC-205$^+$ cells were (**c**) CD11c$^+$, and (**d**) BDCA-1$^+$. There were many CD11c$^+$ cells that were not positive for these two markers in the psoriatic dermis. Bar = 100 μm.

populations. Blood monocytes expressed similar levels of CD11c and HLA-DR as CD11c$^+$ BDCA-1$^-$ psoriatic dermal DCs (data not shown), but were 99% CD14^{hi} (Figure S2b, row 3, black line no fill) unlike CD11c$^+$ BDCA-1$^-$ psoriatic dermal DCs, which were CD14^{mid}. CD16 FcγRIIIa expression was similar in all three populations. Thus, psoriatic CD11c$^+$ BDCA-1$^+$ dermal DCs are phenotypically mature and closer to normal resident CD11c$^+$BDCA-1$^+$ dermal DCs, whereas psoriatic CD11c$^+$BDCA-1$^-$ cells share phenotypic features of both DCs and monocytes.

Allo-MLR

To test the immunostimulatory capacity of psoriatic dermal DC populations, we FACS-sorted DCs (CD11c$^+$HLA-DR^{hi}BDCA-1$^+$ or CD11c$^+$HLA-DR^{hi}BDCA-1$^-$) and macrophages (CD163^{hi}) for coculture as stimulators in an allogeneic mixed leukocyte reactions (allo-MLR) (Figure 7). Mature monocyte-derived DCs and T cells alone served as positive and negative controls, respectively. Figure 7 shows a representative experiment ($n = 3$). A total of 73.2% of living T cells stimulated with mature monocyte-derived DCs at a stimulator/responder ratio of 1:50 on day 8 post-sorting had undergone extensive proliferation (Figure 7b). MLRs without a stimulator population (T cells) alone had 2.5% background proliferation. CD163^{hi} cells were not more immunostimulatory than T cells alone (2.3%), whereas both CD11c$^+$BDCA-1$^+$-resident DCs and CD11c$^+$BDCA-1$^-$ inflammatory DCs were similarly immunostimulatory (55.3% and 64.5%, respectively). Bulk psoriatic dermal single cell suspensions stimulate 60% (data not shown). These results suggest that CD163^{hi} cells in psoriasis are nonimmunostimulatory macrophages expressing low-level CD11c (Figure 4e), and that although CD11c$^+$BDCA-1$^-$ are phenotypically less mature than the CD11c$^+$BDCA-1$^+$ cells, they are comparably immunostimulatory.

Induction of intracellular cytokines in T cells. We recently demonstrated the presence of Th17 cells from the dermis of psoriatic plaques (Lowes *et al.*, 2008). We now show that CD11c$^+$ BDCA-1$^+$ and CD11c$^+$ BDCA-1$^-$ psoriatic dermal DCs induce IL-17 production in allogeneic CD4$^+$ T cells. Psoriatic dermal émigrés ($n = 3$) were sorted as previously described into CD11c$^+$ BDCA-1$^-$ DCs, CD11c$^+$ BDCA-1$^+$ DCs, or CD163$^+$ macrophages and mixed with normal donor T cells at a 1:10 stimulator:responder ratio. Normal skin dermal émigrés ($n = 2$) were sorted into BDCA-1$^+$ and CD163$^+$ populations. Cells were cultured for 9 days before analysis of T-cell intracellular cytokines and IL-17 and IFN-γ cytokine production in the supernatant. Figure 8 shows a representative experiment.

There were few T-cells positive for IL-17 or IFN-γ when the T cells were cultured alone (Figure 8a, left), with marked increases when cells were stimulated with CD3/28 beads (Figure 8a, right). Culture of the dermal single cell suspensions alone, without addition of the allogeneic T cells, gave similar results to T cells alone, indicating the capability of the syngeneic T cells in the suspension (Figure 8b). There was marked increase in the intracellular cytokine staining when the dermal DCs were cultured with allogeneic T cells (Figure 8c), much more so for the psoriasis lesions. We particularly noted the increase of the IL-17$^+$IFN-γ$^+$ cells induced by the psoriatic DCs, from 0.2 to 12.1%. The psoriatic dermal single-cell suspensions also induced more IL-17 and IFN-γ protein than normal skin (Figure 8d). This was similar to controls of T cells alone and to T cells stimulated with CD3/CD28 (data not shown).

The psoriatic BDCA-1$^+$ and BDCA-1$^-$ populations were able to induce similar percentages of these IL-17$^+$IFN-γ$^+$ cells, although less than the bulk dermal single cell suspensions (Figure 8e and f, right). This reduction is likely due to the process of FACS-sorting, as sorting reduces the ability of the DCs to polarize T cells by an average of sixfold (analysis of the ability of bulk single-cell suspensions before and after sorting, data not shown). Also, it was technically difficult to titrate an exact DC:T cell ratio in bulk émigrés, making it difficult to compare results with pure sorted cells. The BDCA-1$^+$ DCs from normal skin did not induce IL-17$^+$IFN-γ$^+$ cells (Figure 8e, left), nor did the normal or psoriatic skin macrophages (Figure 8g).

In summary, normal skin dermal émigrés polarized few Th17 cells, none of which were IL-17 and IFN-γ producing, whereas psoriatic bulk émigrés and dermal DCs (BDCA-1$^+$ and BDCA-1$^-$) polarized Th17 cells producing both IL-17 and IFN-γ. CD163$^+$ macrophages from either normal or psoriatic skin were not able to polarize T cells to produce IL-17.

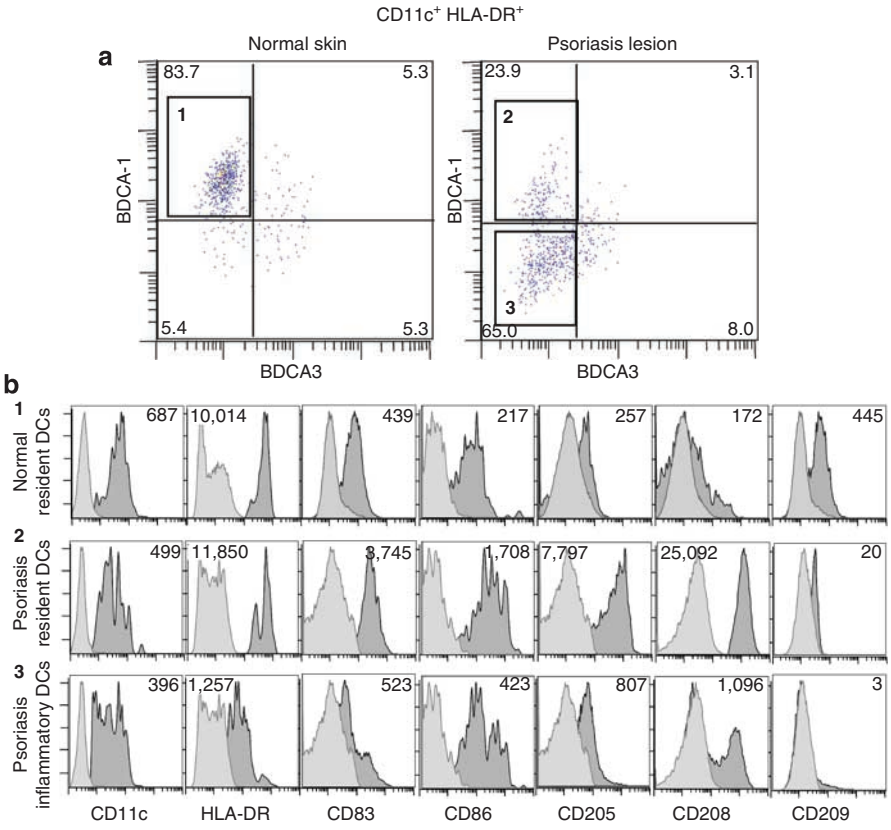

Figure 6. Psoriatic inflammatory dermal DCs (CD11c$^+$ BDCA-1$^-$) are less mature than resident BDCA-1$^+$ dermal DCs. Flow cytometric analysis of single-cell suspensions of dermal émigrés from normal dermis or psoriatic dermis ($n=3$ for each). (**a**) Large cells gated on CD11c$^+$ HLA-DR^{hi}. In normal dermis, most myeloid DCs were BDCA-1$^+$(Box 1). In psoriatic dermis, myeloid DCs were either BDCA-1$^+$ (Box 2) or BDCA-1$^-$ (Box 3). Percent of myeloid cells indicated for each quadrant. (**b**) Histograms in each row were gated on boxes (1–3) as identified above in Figure 6a. Dark gray histogram represents antigen expression, light gray represents isotype. Median fluorescence intensity is indicated in the upper right or upper left corner of each histogram. Resident BDCA-1$^+$ myeloid DCs from normal and psoriatic dermis were phenotypically similar, whereas the additional population of BDCA-1$^-$ DCs in psoriasis showed lower HLA-DR and were smaller cells. Psoriatic BDCA-1$^+$ DCs demonstrated the highest expression of DC-LAMP/CD208, and DEC-205/CD205.

DISCUSSION

In psoriasis as well as normal skin, myeloid DCs identified by BDCA-1 were present, and their numbers remained the same during a course of etanercept therapy. These resident BDCA-1$^+$ DCs were often in clumps in the dermis and were positive for mature markers such as DC-LAMP and DEC-205. In psoriasis, there was a marked increase in CD11c$^+$ cells currently best identified as BDCA-1$^-$ and we have termed these BDCA-1$^-$ dermal cells as ''inflammatory'' myeloid DCs. This group of inflammatory cells may be heterogeneous, including the Tip-DCs, IL-20-producing DCs (Wang *et al.*, 2006), and IL-23-producing DCs (Lee *et al.*, 2004; Zaba *et al.*, 2007a). The success of anti-TNF therapies to reduce all these cytokines and mediators and reverse the psoriatic phenotype supports the potential pathogenic role of these DCs (Zaba *et al.*, 2007a). A similar finding was recently described in normal and diseased kidney, with a stable number of BDCA-1$^+$ cells but increased DC-SIGN$^+$ cells during inflammation (Woltman *et al.*, 2007).

We have interpreted our *in situ* data to indicate that there is a distinction between BDCA-1$^+$ and BDCA-1$^-$ myeloid DCs. However, there was little difference in the function of these two FACS-sorted populations *ex vivo*, as both BDCA-1$^+$ and BDCA-1$^-$ DCs were immunostimulatory in the allo-MLR and induced Th1, Th17, and a mixed Th1/Th17 cell type. We interpret these findings as confirmation of their DC function, and an indication that the CD11c$^+$BDCA-1$^-$ cells are not monocytes or macrophages (which cannot stimulate in these tests). Their ability to perform in these assays indicates their antigen-presenting potential, rather than being a true characterization of their role *in situ* during inflammation. This lack of difference in their function is perhaps surprising: we had expected the BDCA-1$^-$ population to be less stimulatory. The similar function of these two populations may be due to maturation of the BDCA-1$^-$ cells during migration out of the dermis, demonstrated by acquisition of DC-LAMP by a proportion of BDCA-1$^-$ cells in the single cell suspensions, with concomitant upregulation of DC-defining functions. Thus, although there are several limitations to this system, it is currently the only method available to study the potential functions of these cells. Another possibility for the similar capability of the BDCA-1$^+$ versus the BDCA-1$^-$ populations is that the CD11c$^+$BDCA-1$^-$ FACS-sorted cells contain a small number

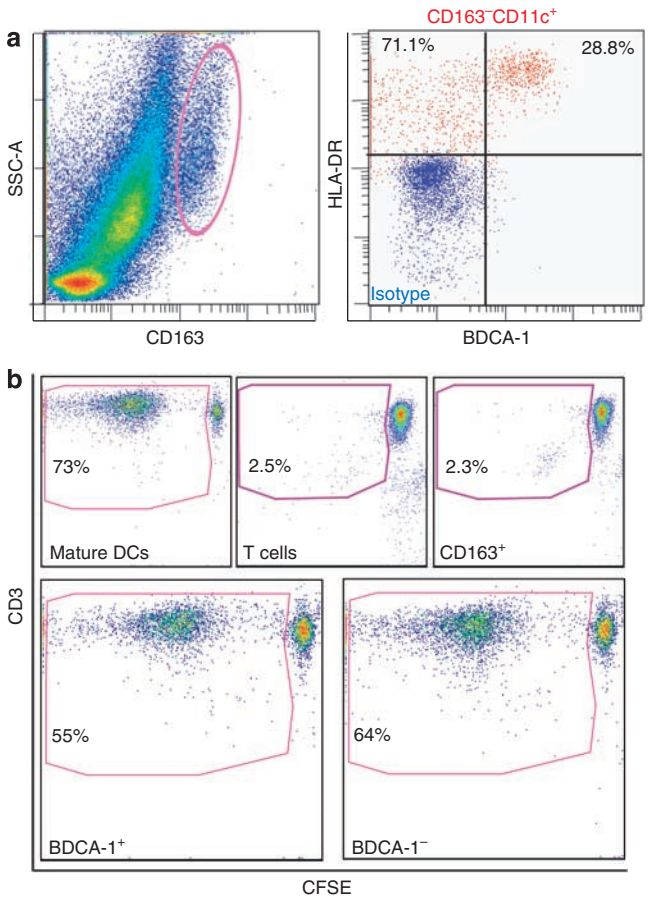

Figure 7. Both psoriatic CD11c$^+$BDCA-1$^+$ resident DCs and CD11c$^+$BDCA-1$^-$ inflammatory DCs were immunostimulatory in an allo-MLR. Single-cell suspensions of psoriatic dermal émigrés were sorted into (**a**, **left**) CD163^{hi} or (**a**, **right**) CD3$^-$CD45$^+$HLA-DR^{hi}CD11c^{hi}BDCA-1$^+$ or CD3$^-$CD45$^+$HLA-DR^{hi}CD11c^{hi}BDCA-1$^-$ compared with isotype (red). (**b**) Gate contains CD3$^+$ proliferating T cells with left-shifted CFSE as cells proliferated. Positive control (monocyte-derived mature DCs) on day 8 postsorting at 1:50 stimulator:responder ratio. Background proliferation of T cells alone (2.5%); CD163^{hi} cells did not stimulate above background. BDCA-1$^+$ and BDCA-1$^-$ cells stimulated T-cell proliferation similarly (both >55%). Representative graphs from three experiments.

of immunostimulatory BDCA-3$^+$ cells. However, there are too few BDCA-3$^+$ cells to separate for functional studies, and in future sorting strategies, we will exclude these cells.

There are several possible mechanisms to explain an increase in inflammatory myeloid DCs in psoriasis lesional skin, including arising from *in situ* DCs in nonlesional skin, from circulating DC precursors or monocytes. These cells may arise from BDCA-1$^+$-resident myeloid DCs that down-regulate their BDCA-1 surface expression, or even Langerhans cells. This is supported by the xenotransplant psoriasis model, where human transplanted nonlesional skin from psoriasis patients is grafted onto immunocompetent mice and the grafts become psoriatic without further intervention (Nestle *et al.*, 2005). In this model, resident cells in the nonlesional skin are sufficient and capable of inducing the psoriatic phenotype, without any contribution from circulating cells. However, lesional skin immunocytes may behave differently in this xenotransplant system and may take on unconventional roles.

Secondly, these inflammatory myeloid DCs may arise from peripheral blood DC precursors, including hematopoietic stem-cell precursors, multipotent progenitor, common myeloid precursor, circulating pre-DC (CD11c$^+$ HLA-DR^{hi}, CD16$^+$) or monocytes (Randolph *et al.*, 2002; Serbina *et al.*, 2003; Tacke and Randolph, 2006; Massberg *et al.*, 2007; Piccioli *et al.*, 2007). It is possible that any of these precursor cells are ''pre-inflammatory DCs'', migrating into the skin in response to a chemokine gradient or other stimulus, supported by our observation of a reduction of some circulating DC subsets in psoriasis blood compared with normal. The concept that the inflammatory DCs arise from circulating precursors rather than *in situ* DCs is also consistent with other murine models, demonstrating that during steady state, Langerhans cells and dermal DCs are able to locally proliferate, but during active inflammation there is migration of peripheral blood DC precursors into the skin (Bogunovic *et al.*, 2006; Ledgerwood *et al.*, 2008; Liu *et al.*, 2007; Massberg *et al.*, 2007).

CD11c$^+$BDCA-1$^-$ dermal DCs retain some phenotypic features of their likely peripheral blood precursors, including smaller size, low-level expression of CD14, and CD163—supporting the concept that these cells migrate into the skin from the blood. CD163^{hi} cells, however, when sorted from psoriasis dermis are not immunostimulatory, thus they can be distinguished functionally from the immunostimulatory CD11c$^+$BDCA-1$^-$ DC population. Other phenotypic markers that have been attributed to both inflammatory DCs and inflammatory macrophages include CD68 (Wang *et al.*, 2006), CD32 (Dhodapkar *et al.*, 2007), and DC-SIGN/CD209 (Granelli-Piperno *et al.*, 2005). The colocalization of some of these antigens may also be due to plasticity between these immature DC and macrophage populations within the tissue during inflammation.

In addition to characterization of psoriatic myeloid dermal DCs, this article presents evidence for Th17 polarization by psoriatic DCs. Both psoriatic CD11c$^+$BDCA-1$^+$-resident DCs and CD11c$^+$BDCA-1$^-$ DCs had the capacity to polarize Th17 cells, although most polarization was induced by bulk psoriatic dermal cells not damaged by sorting. Moreover, bulk psoriatic émigrés induced Th17 cells producing both IL-17 and IFN-γ compared with normal skin. As psoriasis is now thought of as a mixed Th17/Th1 disease with strong IL-17 and IFN-γ signatures (Blauvelt, 2007; Ghoreschi *et al.*, 2007; Lowes *et al.*, 2007), it is possible that these IL-17/IFN-γ-positive T cells induced by psoriatic DCs are pathogenic. However, Th17 cell induction could also be due to an allo-response, so future studies are planned to evaluate autologous effects of these DCs, and also to study other skin diseases to assess psoriasis-specific effects.

In summary, we have demonstrated a marked increase in CD11c$^+$BDCA-1$^-$ myeloid DCs in psoriasis, and this group of inflammatory DCs contains Tip-DCs. These cells are immunostimulatory and capable of Th17 polarization, but their most essential contribution may be to secrete inflammatory products such as iNOS, TNF, IL-20, and IL-23. We

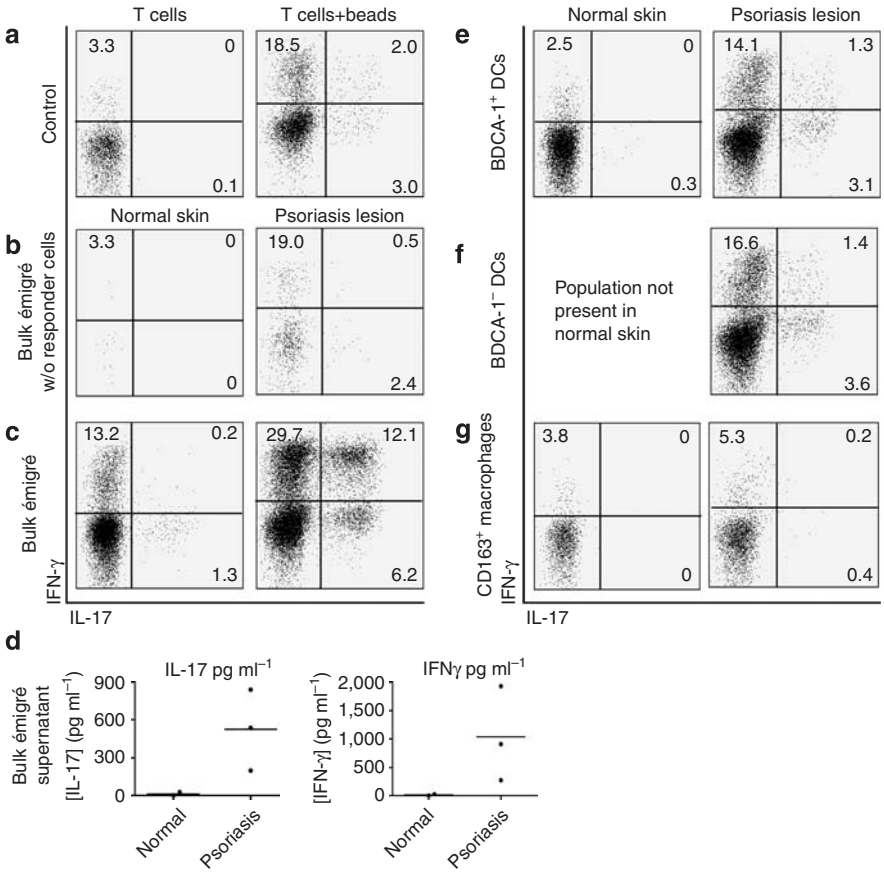

Figure 8. Psoriatic dermal DCs induce IL-17/IFN-γ-producing T cells. Allogeneic T-cell responders were mixed with FACS-sorted stimulator populations described in Figure 6, or with bulk émigrés from either normal or psoriatic dermal skin, at 1:10 ratio for 9 days. Intracellular cytokine expression of T cells (live $CD3^+CD4^+CD8^-$ cells) measured by (**a**–**c**, **e**–**g**) flow cytometry or (**d**) protein supernatant. (**b**, **c**, **e**–**g**) Comparison of T-cell polarization of normal dermal DCs (left panels) with psoriatic dermal DCs (right panels). (**a**) Controls: T cells alone (left panel) demonstrated baseline intracellular cytokine expression and T cells + beads (right panel) measured intracellular cytokine expression following CD3/CD28 bead stimulation. (**b**) Bulk émigrés without responder T cells measured baseline T-cell cytokine production in single-cell suspensions. Other stimulator populations mixed with responder T cells were (**c**) unsorted bulk émigrés, (**e**) sorted $HLA\text{-}DR^+CD11c^+BDCA\text{-}1^+$ dermal DCs, (**f**) sorted $HLA\text{-}DR^+CD11c^+BDCA\text{-}1^-$ dermal DCs, or (**g**) sorted $CD163^+$ macrophages. Psoriatic bulk dermal DCs induced a population of IL-17/IFN-γ-producing T cells, as did $BDCA\text{-}1^+$ and $BDCA\text{-}1^-$ DCs (although to a lesser degree, possibly due to sorting). Quadrant percentages are indicated by representative graphs from three experiments. (**d**) Supernatant of the same cultures was collected for analysis of cytokine protein expression. Bulk psoriatic dermal émigrés mixed with allogeneic T cells induced IL-17 and IFN-γ production, whereas normal dermal DCs did not.

hypothesize that the resident $BDCA\text{-}1^+$ are likely the myeloid DCs capable of classic antigen presentation to cutaneous T cells, although their dermal location and organization into clumps associated with T cells suggests that this function may occur within the skin environment (''ectopic lymphoid tissue'') rather than in an extracutaneous lymphoid organ such as a lymph node (Lew *et al.*, 2004). In contrast, although $BDCA\text{-}1^-$ DCs are certainly capable of robust antigen presentation in an allo-MLR and have the ability to polarize T cells (DC-defining characteristics), their main role may actually be as an inflammatory mediator production house, amplifying and maintaining psoriatic inflammation. Further studies need to be performed to prove this, dependant on new tools to study DCs *in situ*. We also need new markers to identify these inflammatory DCs in a positive manner, rather than as a negative population. We need to understand how and where these nonresident $CD11c^+BDCA\text{-}1^-$ DCs are generated, to be able to specifically shut down their production of pro-inflammatory mediators and bring about rapid resolution of clinical disease.

MATERIALS AND METHODS

Skin samples

Skin punch biopsies (6 mm diameter) were obtained from normal volunteers and psoriasis patients under a Rockefeller University IRB-approved protocol. Informed consent was obtained and the study was performed in adherence with the Declaration of Helsinki Principles. Lesional and nonlesional samples used for immunohistochemistry and immunofluorescence were from patients enrolled in an etanercept clinical trial as described previously (Zaba *et al.*, 2007a). Biopsies were frozen in OTC (Sakura, Tokyo, Japan) and stored at −80 °C until required. Dermal single cells suspensions from normal human skin were obtained from waste abdominoplasty skin as described previously (Zaba *et al.*, 2007b). Dermal single-cell suspensions from psoriasis shave biopsies

were obtained following overnight incubation in dispase (Invitrogen Life Technologies, Carlsbad, CA) 1 mg ml^{-1} at 4 °C, to separate the epidermis. The dermis was cultured for 36–48 hours at 37 °C in RPMI 1640 (Gibco-BRL Life Technologies, Carlsbad, CA) supplemented with 10% pooled human serum (Mediatech Inc. Manassas, VA), 0.1% gentamicin reagent solution (Gibco-BRL Life Technologies), and 1% 1 M HEPES buffer (Sigma, St Louis, MO) (Lowes *et al.*, 2005).

Antibodies

All antibodies used for immunohistochemistry, immunofluorescence, and FACS are listed in Supplementary Table 1a and b.

Immunohistochemistry

Skin sections were stained as described previously (Zaba *et al.*, 2007b). Positive cells per mm were counted manually using computer-assisted image analysis (NIH IMAGE 6.1).

Immunofluorescence

Skin sections were stained as described previously (Zaba *et al.*, 2007b). Images were acquired using appropriate filters of a Zeiss Axioplan 2I microscope with Plan Apochromat 20 × 0.7 numerical aperture lens and a Hagamatsu orca ER-cooled charge-coupled device camera, controlled by METAVUE software (Universal Imaging, Ypsilanti, MI). Images in each figure are presented both as single color stains (green and red) located above the merged image, so that one can appreciate the localization of two markers on similar or different cells. Cells that coexpress the two markers in a similar location are yellow in color. A white line denotes the dermoepidermal junction. Dermal collagen fibers gave green autofluorescence, and antibodies conjugated with a fluorochrome often gave background epidermal fluorescence.

FACS

Cells from dermal cell suspensions or from whole blood were stained with the antibodies listed in Table 1b. Briefly, cells were stained for 20 minutes at 4 °C, whole blood samples were lysed with FACSlyse (BD Biosciences, San Jose, CA) for 20 minutes, all samples were washed with FACSwash (PBS 0.1% sodium azide and 2% FBS) (BD Biosciences), and re-suspended in 1.3% formaldehyde (Fisher Scientific, Pittsburgh, PA) in FACSwash. For intracellular cytokine staining assays, cells were incubated in aqua marina live/dead dye (BD Biosciences) for 30 minutes on ice, washed, fixed with 4% paraformaldehyde (BD Biosciences) for 20 minutes on ice, blocked in 1:100 mouse serum (BD Biosciences), permeabilized in FACSPerm (BD Biosciensces), incubated simultaneously with intracellular cytokine and extracellular antibodies, washed, and collected. All samples were acquired using an LSR-II (BD Biosciences) and analyzed with FlowJo (Treestar, Ashland, OR). Appropriate isotype controls were used.

FACS sort and MLR

This was performed as described previously (Zaba *et al.*, 2007b; $n = 3$). Dermal cells from single cell suspensions of psoriatic lesional skin were stained with CD3, CD45, CD11c, HLA-DR, BDCA-1, and CD163 antibodies (Table 1b), and sorted on a FACSAria (BD Biosciences) using a low-pressure setting. Three populations were obtained: CD163$^+$ macrophages, CD3$^-$CD45$^+$CD11c$^+$HLA-DR$^+$BDCA-1$^+$ DCs (''resident myeloid dermal DCs''), and CD3$^-$CD45$^+$CD11c$^+$HLA-DR$^+$BDCA-1$^-$ DCs (''inflammatory myeloid dermal DCs''). Responding T cells were obtained from a normal volunteer by density centrifugation over Ficoll-Paque Plus (Amersham Biosciences), followed by T-cell purification using a T-cell-negative selection kit (Dynal, Carlsbad, CA). Sorted stimulator populations were cocultured with 10 μm carboxy fluoroscein succinimidyl ester (CFSE)-labeled T cells at a 1:50 ratio, and T-cell proliferation was analyzed on day 8 post-sorting. Proliferation assays were harvested, stained with 250 ng ml^{-1} propidium iodide to label dead cells, and CD3-APC for 15 minutes at room temperature. Propidium iodide-negative cells were gated and then plotted as CFSE versus CD3$^+$ cells, where proliferating cells diluted their content of CFSE and move to the left of the nonproliferating cells. The CFSE-low cells were quantified as a percentage of live cells in the culture. CFSE-labeled T cells alone were used as negative control, and coculture with monocyte-derived mature DCs were used as positive control. The process for making mature DCs has been described previously (Lee *et al.*, 2002).

T-cell polarization assays

Stimulator DC populations were prepared as above for the MLR ($n = 3$ for psoriasis dermis; $n = 2$ for normal dermis). Unlabeled bead-separated (Dynal) T cells were used as responding cells. Stimulator and responder cells were cultured at a ratio of 1:10. for 9 days. Cells were then resuspended in RPMI media and activated for 4 hours using 25 ng ml^{-1} phorbol myristate acetate and 2 μg ml^{-1} ionomycin, in the presence of 10 μg ml^{-1} brefeldin A (all Sigma-Aldrich St Louis, MO) at 37 °C. Unactivated controls were treated with brefeldin A only. Ethylenediaminetetraacetic acid (2 mM; Fisher Scientific) was added for 10 minutes at 37 °C to stop activation. T-cell intracellular cytokine expression was analyzed using the following antibodies: CD3, CD4, CD8, IFN-γ, IL-17, and aqua live-dead stain. T cells alone were used as a negative control, and T cells stimulated with CD3/28 beads (Dynal; 1/2 bead per T cell) were used as a positive control. Before activation, T-cell polarization assay plates were centrifuged and the supernatant collected for measurement of cytokine protein concentration using the human cytokine 25-plex kit (Invitrogen, Carlsbad, CA) on a luminex CS1000 autoplex analyzer (Luminex corporation). The assay was performed in duplicate according to the manufacturer's instruction, and results were averaged.

Statistical analysis

Two-tailed paired Student's *t*-test was used to compare normal versus psoriasis nonlesional and lesional cell counts for BDCA-1$^+$, BDCA-3$^+$, and CD11c$^+$BDCA-1-BDCA-3$^-$ DC populations. The two-tailed *P*-values are designated as $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1a. Antibodies used for immunohistochemistry and immunofluorescence.

Table S1b. Antibodies used for flow cytometry.

Figure S1. Additional characterization of myeloid DCs in skin and blood in healthy volunteers and psoriasis patients.

Figure S2. Additional FACS analysis of myeloid DC populations from normal and psoriasis single-cell suspensions.

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